NATURAL POLYMER BASED (ALGINATE AND CHITOSAN) MICROPARTICLES FOR ORAL DRUG DELIVERY

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Life science

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CERTIFICATE

This is to certify that the thesis entitled "NATURAL POLYMER BASED (ALGINATE AND CHITOSAN) MICROPARTICLES FOR ORAL DRUG DELIVERY" submitted to National Institute of Technology; Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by Pravat Kumar Parida(409LS2048) under my supervisions and guidance.

Dr. (Miss.) B.Nayak
Advisor

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DECLARATION

I hereby declare that the thesis entitled **“N**atural polymer based (alginate and chitosan) microparticles for oral drug delivery**”**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bona fide and original research work carried out by me under the guidance and supervision of Dr. (Miss.) Bismita Nayak, Assistant Professor, Department of Life Science , National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:

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Date:

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ABSTRACT

Absorption of proteins into cationic chitosan microparticles through electrostatic interaction is a common process suitable for oral delivery of proteinaceous drugs. In this research work, in order to achieve a good stability and encapsulation efficiency for an oral drug delivery system, different combinations chitosan, acetic acid Sodium tripolyphosphate and model protein bovine serum albumin were tried and formulated. Then alginate microparticles were coated with the BSA loaded chitosan. Morphological characterizations of the particles were done using zeta sizer and SEM (scanning electron microscope). It was found that Chitosan of 4mg/ml and 5mg/ml concentration had loading efficiency more than 60% and with a particle size in between 400-500 nm. Again when the particles were coated with alginate, the particle size increased from 1400-1600nm (1.4-1.6 μm), can be effectively used for oral drug delivery. *In vitro* release of BSA from chitosan microparticles and BSA loaded alginate coated chitosan particles were checked by taking particles at different time intervals at pH7.4 using PBS (phosphate saline buffer). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assay shows that encapsulation with chitosan and further coating with alginate could effectively protect BSA from degradation or hydrolysis in acidic condition for at least 2 hours (using HCl pH 2.0).

Oral delivery of peptides and proteins remains an attractive alternative to parenteral delivery and has challenged various attempts at drug delivery development. Incorporation of new tools into the delivery systems that can raise membrane permeability of macromolecules is essential to attain high oral bioavailability that is acceptable in clinical applications. Administering drugs orally is so far the most widely used route of administration, although it is generally not suitable for peptide and protein drugs. The main reasons for the low oral bioavailability of biological are presystemic enzymatic degradation and poor penetration of the intestinal membrane. Much has been learned in the past few decades about macromolecular drug absorption from the gastrointestinal (GI) tract, including the barriers that restrict GI absorption. Various strategies have been followed to overcome such barriers and to develop safe and effective oral delivery systems for proteins. Most oral delivery strategies for biological are based on system equipped to protect against enzymatic degradation and provide high transfer of drugs across the epithelial mucosa. Certain particles can be taken up by the Payer's patches without employing additional penetration enhancers. So far, polymeric drug delivery systems based on hydrogels, nanoparticles, microspheres, and lipid-based drug delivery systems (e.g. micro-emulsions, liposomes, and solid lipid nanoparticles) have been developed and employed for oral macromolecular drug delivery. Natural polymers like chitosan, alginate etc. have caught the eyes of the pharmaceutical scientists till now for the purpose of the oral drug delivery due to their attractive features like non toxicity and biodegradable properties. Chitosan, a natural cationic polysaccharide derived from chitin is a copolymer of glucosamine and N-acetyl glucosamine units (Ravi Kumar, 2007), has gained an increased attention in biomedical as well as pharmaceutical purposes due to its biocompatible properties such as non-toxicity, biodegradability (George and Abraham, 2006 and Gan *et al*., 2005), muco-adhesive properties etc. This biodegradable polymer is synthesised by alkaline deacetylation of chitin by the enzyme Chitin deacetylase as presented in figure 1.

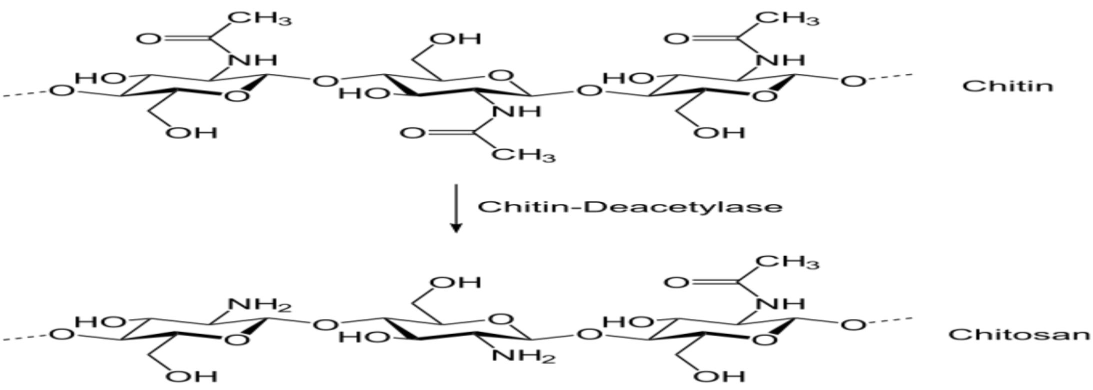

Fig.1 Production of chitosan by the deacetylation of chitin by the enzyme chitin deacetylase.

On the other hand Alginate (Fig.2) is an anionic polysaccharide extracted from brown algae, composed of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues, arranged in homopolymeric blocks of each type (MM, GG) and heteropolymeric blocks (MG) (Takka and Gruel, 2010) increase the stability of drugs and prevent an immediate desorption of drugs from chitosan carriers in gastrointestinal tract (GI) tract.

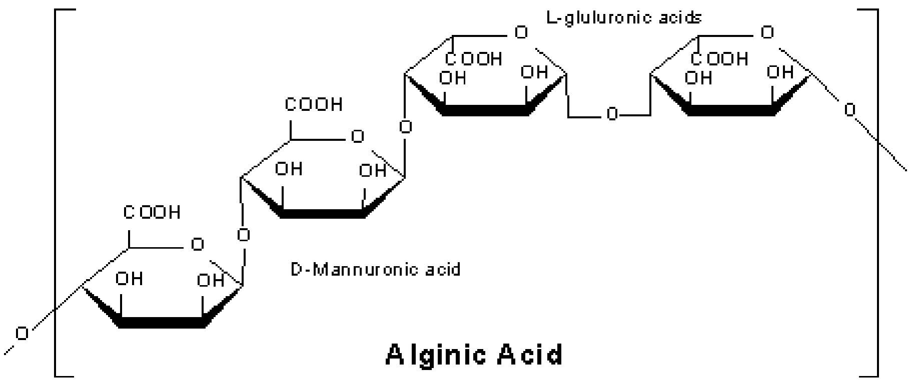

Fig.2 Structure of alginic acid (alginate).

Both the polymers have been used extensively in production of pharmaceutical products especially for drug delivery purposes. In this paper chitosan and alginate were chosen due to their extensive biocompatible properties. Various authors used Alginate to coat over BSA loaded chitosan for maintaining stability of the drug and chitosan for good drug encapsulation properties. Keeping this in view we have used both alginate and chitosan for the formulation of drug delivery system in this work.

Peptides and proteins have become the drugs of choice for the treatment of numerous diseases as a result of their incredible selectivity and their ability to provide effective and potent action (Frokjaer and Otzen, 2005) due to their fewer side effects and higher potency to cure diseases. Pharmaceutical scientists have produced variety of protein and peptide drugs in commercial scale using biotechnological techniques (Shah *et al*., 2002, Torchilin *et al.*, 2003 and Frokjaer and Otzen, 2005). In recent years, there has been a significant increase in the number of targeting mechanisms available to the pharmaceutical scientist to provide site-specific delivery in the gastrointestinal (GI) tract (Hwang *et al*., 1998). Generally, it is highly beneficial to target a drug to a particular site within the GI tract either to maximize a therapeutic response or to reduce side effects caused by drug delivery to an inopportune region of the gut (Hwang *et al*., 1998 and Lee *et al.*, 1991). Drug absorption differences in various gastrointestinal segments. In general, drug absorption is moderately slow in the stomach, rapid in the small intestine, and sharply declining in the large intestine.

Compensation for changing absorption characteristics in the gastrointestinal tract may be important for some drugs. For example, it is rational for a delivery system to pump out the drug much faster when the system reaches the distal segment of the intestine, to avoid the entombment of the drug in the feces (Burnside, 2004). The instability and poor absorption of drugs in gastrointestinal tract is major obstacles in the development of oral drug delivery system for mucosal vaccine as well as protein and peptide drugs. Problems such as pre-systemic enzymatic degradation (Morishita and Peppas, 2006) and acid degradation in stomach, poor permeability across the gastrointestinal mucosa and the first-pass metabolism greatly limited the uptake of antigens by M-cell which is very important step for immune response (George and Abraham, 2006 and Jepson *et al.*, 2004). Thus to overcome such problems different strategies like liposomes (Wang, 1996; Okada *et al*.,1997 and Anderson *et al.*, 2006), micro/nanoparticles (Vila *et al.*, 2002; Lubben *et al*., 2002 and Lubben *et al.*, 2003), micro/nanoemulsion (Bielinska, 2007), and etc., have been explored to encapsulate antigens for the mucosal vaccine.

In developing oral protein delivery systems with high bioavailability, three practical approaches might be most helpful: (1) modification of the physicochemical properties of macromolecules; (2) addition of novel function to macromolecules; or (3) use of improved delivery carriers (Morishita and Peppas, 2006).

Chitosan and alginate in oral drug delivery

In design of oral delivery of peptide or protein drugs, one important aspect is that some natural polymers like alginate and chitosan (CS) have attracted increasing attention since most of the synthetic polymers are immunogenic and the incorporation of proteins into these polymers require a harsh environment which may denature and inactive the desired protein (George and Abraham, 2006).

Chitosan(CS) micro/nanoparticles can be easily prepared by ionic gelation method using tripolyphosphate (TPP) as precipitating agent (Gan *et al.,* 2005).In spite of all superior qualities like non-toxicity, biodegradability etc. chitosan has an apparent pKa of 5.6 and is only soluble in acidic solutions. When incubated in physiological fluid environment, chitosan will lose its capacity of mucoadhesive properties and permeation enhancing effect due to the de-protonation of chitosan, which would make chitosan carriers lose its advantage compared with other carriers for mucosal vaccine (Xing *et al.,* 2008).

There are numerous factors affecting the relevant properties of the alginate–chitosan capsules (George & Abraham, 2006). Among these factors are the composition, molecular weight and deacetylation degree of chitosan (Alsarra *et al.,* 2002). While pure CS is soluble in low pH and insoluble in higher pH, alginate shows the opposite trend. Upon mixing, the ammonium groups of chitosan and the carboxylate groups of alginate ionically interact to form the polyelectrolyte complex. Complexation of chitosan with alginate reduces the porosity of gels and decreases the leakage of the encapsulated drugs. The solubility of chitosan at low pH is reduced by the alginate network since alginate is insoluble under low pH conditions, and the possible dissolution of alginate at high pH is reduced by chitosan which is stable at high pH ranges (George and Abraham, 2006). So a stable rigid drug delivery matrix of the alginate–CS polyelectrolyte complex can be produced by the electrostatic interaction between the carboxyl groups of alginic acid and amino groups on CS, which can protect the drug from the harsh acidic environment of the stomach (Wittaya-areekul *et al.*, 2006; Gotoh *et al.*, 2004; Sezer and Akbuga, 1999; Tapia *et al.*, 2007 and Takka and Gruel, 2010).

Chitosan could enhance the immunogenicity of poor immune response antigens in the form of solution and micro/nanoparticles (Zaharoff *et al*., 2007 and Amidi, 2007). Among the various natural polymers available, chitosan (CS) is perhaps one of the most widely used biopolymers for the preparation of nanoparticles (Allemann, 1998). Absorption of antigens onto chitosan microparticles via electrostatic interaction is a common and relatively mild process suitable for mucosal drug delivery.

Alginic acid, also called algin or alginate, is an anionic polysaccharide distributed widely in the cell walls of brown algae, where it, through binding water, forms a viscous gum. In extracted form it absorbs water quickly; it is capable of absorbing 200-300 times its own weight in water (Roew and Raymond, 2009). Its colour ranges from white to yellowish-brown. It is sold in filamentous, granular or powdered forms. Alginate is extracted for commercial purposes from various species of kelp, or brown algae including *Laminaria hyperborean*, *Ascophyllum nodosum*, and *Macrocysis pyrifera* (Smidsrod and Skjak-Braek, 1990). Within the kelp species, alginate is found in the intracellular matrix where it constitutes up to 40% of the dry weight. The alginate forms mixed slats with various cations naturally found in sea water including Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Na^{+}, and the native species is usually found as an insoluble Ca^{2+} cross-linked gel (Sutherland, 1991). This alginate as it has ability to form a gel in the presence of divalent cations such as calcium. This gel shrinks at acidic pH and erodes at alkaline pH. Therefore, it can be used effectively to deliver drug to the intestine (Desai *et al*., 2009).

The natural polymers like alginate have been used to develop drug delivery system for entrapping the delivering drugs orally (Ain *et al*., 2003). When alginate is harvested, the algae is mechanically collected and dried, then the material is milled and treated with dilute acid to remove and dissociate neutral homopolysaccharides and exchange the alkaline earth cations with H^{+} before the alginate in extracted. With the addition of sodium carbonate below pH 10, the alginate is then converted to the soluble sodium salt from the insoluble protonated form and can be further purified and sold in salt or acid form (Sutherland, 1991). Due to the natural extraction process used to obtain alginate, there are many impurities that may potentially contaminate the product. These impurities include heavy metals, endotoxin, proteins, other carbohydrates, and polyphenals contained in the kelp (Smidsrod and Skjak-Braek, 1973). When harvested alginate is used in food and drug industries small traces of theses impurities are acceptable, but when it comes to medicinal applications they must be removed. New methods of harvesting and purification have been developed to address the problem of contamination, and now pharmaceutical grade alginate is available from numerous chemical manufactures. Uncoated calcium alginate gel beads are destabilised when chelators such as phosphate, lactate or citrate or non-gelling cations like sodium or magnesium ions are present (Smidsrod and Skjas, 1990).

Therapeutic proteins and oral drug delivery system

Most therapeutic peptides and proteins are hydrophilic, with Log P values <0. Thus, they would not be expected to follow the trans-cellular route of absorption through passive diffusion (Camenisch *et al.*, 1998). The dimensions of the para-cellular space lie between 10 and 30–50A°, and the para-cellular route is not an option for macromolecular absorption because it is restricted to relatively small hydrophilic molecules that can fit in these spaces (Rubas, *et al*., 1996). In the case of one of the most widely prescribed protein drugs, insulin, evidence of a para-cellular route of absorption was not shown by either morphocytochemical or biochemical analyses (Bendayan *et al.*, 1994). It was demonstrated that insulin adsorbed to the apical membrane and was internalized by certain types of endocytosis (Agarwal and Khan, 2001). Some proteins have been shown to be actively transported across the epithelial lining of the small intestine in membrane-bound vesicles after binding to cell-surface receptors or binding sites (Bastian *et al*., 1999).

There are some interesting evidences that significant amount of protein and peptide (i.e. enough to induce pharmacological effect) can be absorbed if these are protected from proteolytic degradation from proteolytic enzymes in GI tract (Ward et al., 2000). There are two possibilities to overcome the problems associated with the drug delivery in the GI tract the first one is the functional modification of the intestinal epithelial cell physiology or proteolytic enzyme activity and the second one is the protection of the protein or peptide drugs from adverse conditions of GI tract by encapsulating drugs with stabilising agents using pharmacological technologies. A great number of publications reports encapsulation of various molecules such as drugs (Yu *et al*., 2009), proteins (Morishita *et al*., 1992; Pan *et al*., 2002 and Wu *et al*., 2011) etc.

A delivery system with a release profile that is characterized by a time period of no release (lag time) followed by a rapid and complete drug release (pulse release) can be called as an ideal pulsatile drug delivery system. In other words, it is required that a drug should not be released at all during the initial phase of dosage form administration (Saigal *et al*., 2009). Lag time is defined as the time between when a dosage form is placed into an aqueous environment and the time at which the active ingredient begins to get released from the dosage form. While not meant to be limiting, one way to measure lag time is to determine the amount of time before 5% of the drug dose is released from a device when the device is exposed to an appropriate aqueous environment in a United States Pharmacopoeia paddle stirring dissolution apparatus (USP 2) operated at 50 rpm. A lag time of at least 0.5h or longer is considered to be important while a lag time of less than 0.5 h is of little significance. Lag times of more than 4h are desired for delivery of drug into the lower portion of the small intestine while lag times of between 0.5h and 4h are desirable in drug delivery in the upper regions of the gastrointestinal tract (Ayres, 2004).

In the exploration of oral controlled release drug administration, one encounters three areas of potential challenge (Chien, 1992).

1. Development of a drug delivery system: To develop a viable oral controlled release drug delivery system capable of delivering a drug at a therapeutically effective rate to a desirable site for duration required for optimal treatment.

2. Modulation of gastro intestinal transit time: To modulate the GI transit time so that the drug delivery system developed can be transported to a target site or to the vicinity of an absorption site and reside there for prolonged period of time to maximize the delivery of a drug dose.

3. Minimization of hepatic first pass elimination: If the drug to be delivered is subjected to extensive hepatic first pass elimination, preventive measures should be devised to either bypass or minimize the extent of hepatic metabolic effect.

Anatomy and Physiology of the gastrointestinal tract and drug delivery

Systems used for the delivery of therapeutic agents via the oral route must be designed conscious of the physiology of the gastrointestinal tract. The anatomy and physiology of route of administration may dictate many of the requirements for the systems. For example, the device must be able to withstand the saliva, as saliva contains digestive enzymes and other reagents for breaking down whatever is placed in the mouth. The stomach, the main digestive organ of the body, contains many digestive enzymes and very low pH. The pH of the stomach has been measured from 1.4 to 2.1.This harsh environment causes the destruction and denaturation of proteins without protection. The pH of the stomach changes when food is present increasing to nearly 4.0(Dressman *et al*., 1990).

Once through the harsh conditions of the stomach a device reaches the small intestine, which is divided into three regions. The first region, closest to the stomach, is the duodenum, followed by the jejunum and ileum. The duodenum is about 10 inches in length, composes 5% of small intestine and jejunum composes 40% of the length of the small intestine. The entire length of the small intestine is 5 meter and residence time within the organ typically ranges from 2-4 h.

Microparticles smaller than 10 μm are transported to the payer’s patches of the gut associated lymphoid tissue (GALT) (Eldridge *et al.*,1990; Smith *et al*.,1995 and Jani *et al*.,1992). The GALT is represented by the Payer’s patches (PPs), the appendix and small solitary lymphoid nodules. The dome regions of the PPs contains lymphocytes, macrophages and some plasma cells and is covered by the follicle associated epithelium which is specialised in the uptake of antigens from foods. The cells responsible for the actual uptake of viruses, bacteria, toxins and microparticles smaller than 10 μm are the microfold cells (M-cells) (Lydyard and Grossi, 1998; Eldridge *et al*., 1990). These M cells differ in morphology from absorptive cells by their short microvilli, small cytoplasmic vesicles and few lysosomes.Fig.3 gives an overall idea about GIT and protein digesting enzymes involved in GIT.

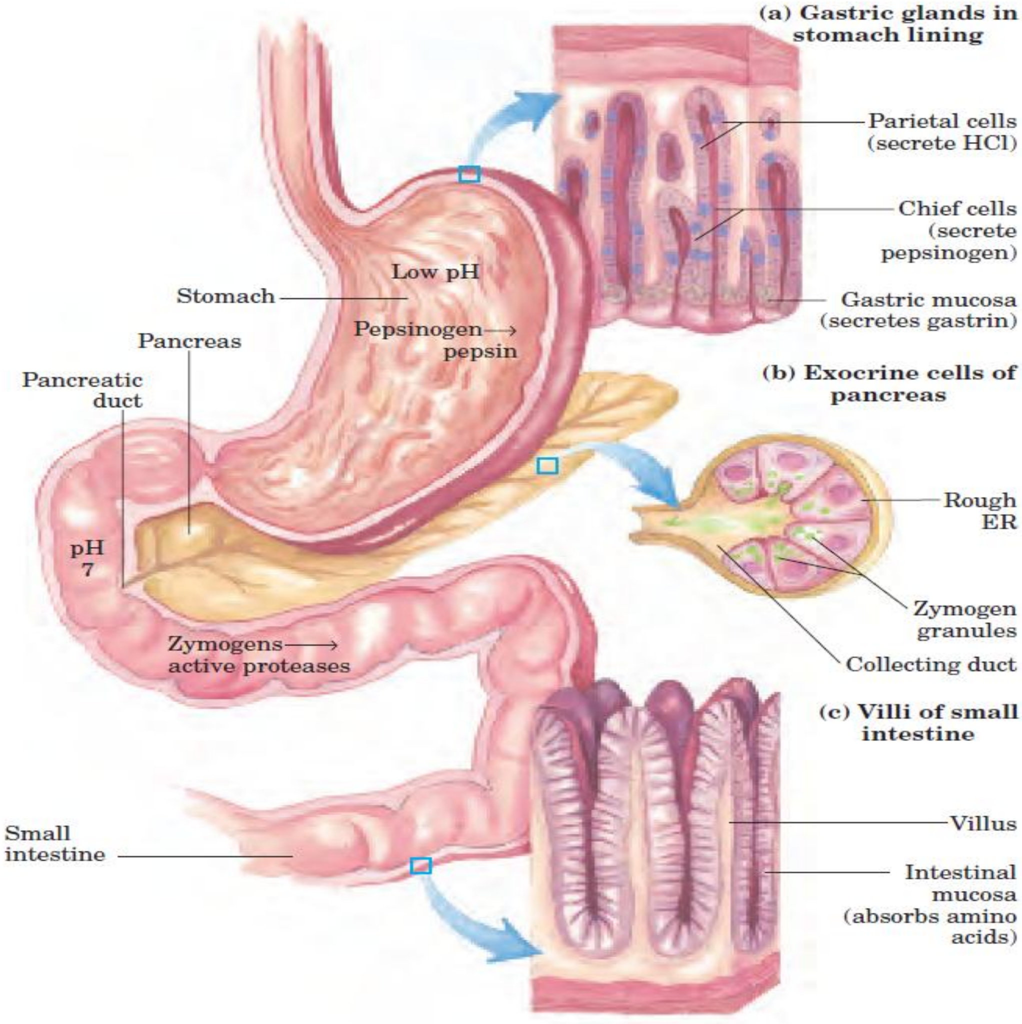

Fig.3. Human gastrointestinal tract (Lehninger principles of Biochemistry, 2008).

The lining of the small intestine are composed of the serous, muscular, areolar, and mucous layers. Only the mucous and areolar layers are the important layers with respect to drug delivery. Transport of the nutrients into the body occurs through the mucious cell layer and into the areolar layer where the nutrients are taken into the blood stream. In the mucosal layer, there are cell layers that stick out of it and into the open areas of the duodenum.

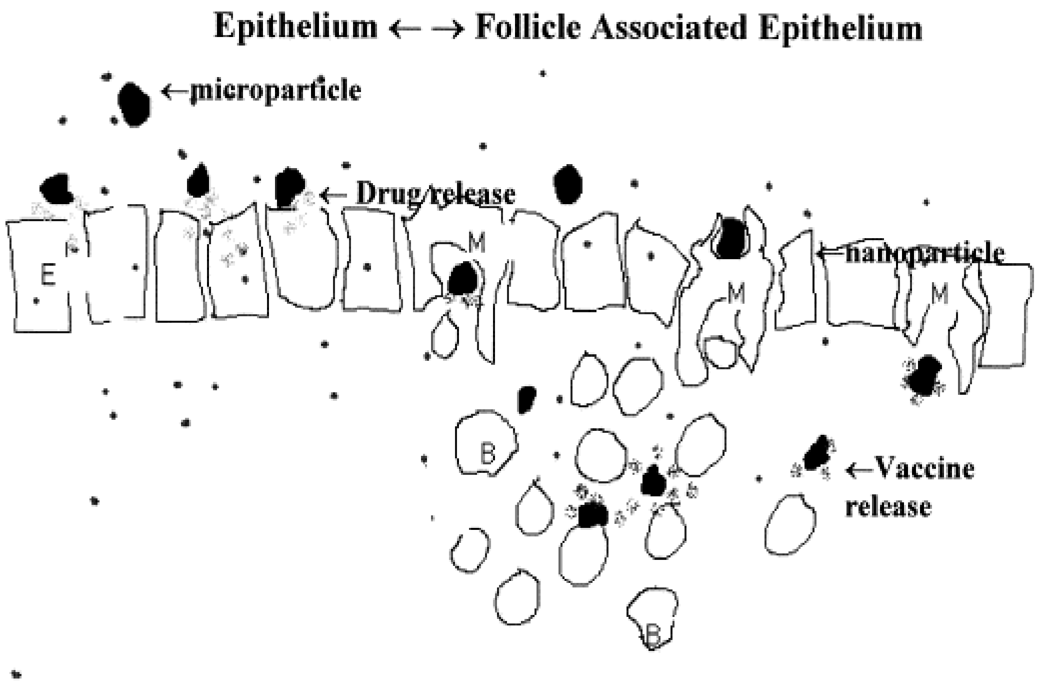

Fig.4. Schematic representation of the follicle associated epithelium. M-cells (M) are located in between the epithelial cells in the lymphoid associated epithelium. M-cells are the entrance port to the Peyer's patches (PP) and efficiently take up microparticles smaller than 10 μm. B, B-lymphocytes; E, epithelial cells (Lubben *et al*., 2001).

Table1. Gastrointestinal Tract: Physical Dimensions and Dynamics (Chien, 1992).

Region	Surface area(m^2)	Length(m)	Transit time	
			Fluid	Digestive solid
GIT	200	----	----	----
Stomach	0.1-0.2	----	50 min	8 h
Small intestine	100-4500	3.0	2-6 h	4-9 h
Large intestine	0.5-1.0	1.5	2-6 h	3h -3days

Model Protein Bovine Serum Albumin

Bovine serum albumin has been reported as a model protein by so many authors for protein drug delivery (Xing *et al*., 2008; Takka and Gruel, 2010; Lemma *et al*, 2006 and Nayar *et al.,* 2010). It has been well characterized and the physical properties of this protein are well known thus it can be used as a model protein. The solution stability of BSA is very good (especially if the solutions are stored as frozen aliquots). In fact, albumins are frequently used as stabilizers for other solubilized proteins (e.g. labile enzymes). However, albumin is readily coagulated by heat (Lewis, 1993). When heated to 50°C or above, albumin quite rapidly forms hydrophobic aggregates which do not revert to monomers upon cooling (Hi-media data).At somewhat lower temperatures aggregation is also expected to occur, but at relatively slower rates. The full-length BSA (bovine serum albumin) precursor protein is 607 amino acids in length. An N-terminal 18-residue signal peptide is cut off from the precursor protein upon secretion; hence the initial protein product contains 589 amino acid residues. An additional 4 amino acids is cleaved to yield the mature BSA protein that contains 585 amino acids (Wright and Thompson, 1975).

Table 2.Basic data about bovine serum albumin fraction-V

Peptide	**Position**	**Length**	**MW**
Full length precursor	1 – 607	607	69,324
Signal peptide	1 – 18	18	2,107
Propeptide	19 – 22	4	478
Mature protein	23 – 607	585	66,776

Physical properties of BSA:

- Number of amino acid residues: 585
- Molecular weight: 66,776 Da
- Isoelectric point in water at 25 °C: 4.8
- Extinction coefficient of 43,824 $M^{-1}cm^{-1}$ at 279 nm

1. First objective is to formulate a cost effective and biocompatible microparticle based oral drug delivery system using natural polymers like alginate and chitosan to overcome the GIT barriers for the delivery of proteinaceous drugs effectively.

2. Chitosan and alginates are the naturally occurring substances with biodegradable and non-toxic properties. Only Chitosan microparticles/nanoparticles as a drug delivery system are not much efficient due to their lesser stability to gastrointestinal tract barriers. Therefore, the second objective is to maintain and enhance the stability of the drug delivery system of chitosan microparticles by coating with alginate.

Preparation of chitosan microparticles

↓

Loading bovine serum albumin (BSA) to chitosan microparticles

↓

Calculation of LC (Loading efficiency) using BCA protein estimation method

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Preparation of alginate coated chitosan microparticles

↓

Measurement of particle size and zeta potential by zeta sizer

↓

Morphological characterization by using scanning electron microscope (SEM)

↓

Study of in-vitro release of protein

MATERIALS

❖Chitosan(Sigma-Aldrich)

❖Sodium alginate(Sigma- Aldrich)

❖Bovine serum albumin fraction –V(Hi-Media)

❖Sodium tripolyphosphate(Sigma-Aldrich)

❖ Calcium chloride

❖BCA™ protein estimation kit (Thermo Scientific)

❖Ultrapure water from Milli-Q water system.

❖Acetic acid

EQUIPMENTS

❖Stratos low-temperature high-speed centrifuge(Thermo, Germany)

❖Freeze Dryer

❖Magnetic stirrer

❖Sonicator

❖Zeta sizer (Malvern)

❖Scanning electron microscope

❖Electrophoretic apparatus

❖Gel documentation system

❖Spectrophotometer

❖SDS-PAGE apparatus

❖Gel documentation system

METHODS

Preparation of chitosan microparticles

❖Chitosan microparticles were prepared by the ionic gelation of chitosan solution with anionic sodium tripolyphosphate.

❖ First, chitosan was dissolved in 1% and 2%(v/v) acetic acid aqueous solution at different concentrations i.e.(2.5,3.0,4.0,5.0,10.0) mg/ml.

❖Then, TPP was dissolved in distilled water at the concentration of 6mg/ml. Subsequently, 5 ml of TPP solution was added drop wisely into 20 ml of

chitosan solution of all concentration. Now chitosan colloid microparticles were formed spontaneously under mild agitation at room temperature on a magnetic stirrer.

- ❖ After 15-20 minutes, chitosan colloid microparticles were centrifuged at 10,000 rpm for 15 min. Then, the supernatant was discarded and the deposit was re-dispersed in distilled water for further use.
- ❖Colloid chitosan microparticles were re-dispersed in 25ml of distilled water under continuous ultrasonication to disaggregate the chitosan microparticles. For each formulation ultrasonication was done for 10 minutes.

Loading bovine serum albumin fraction-V (BSA-V) to chitosan microparticles and calculation of loading efficiency by BCA protein estimation method.

- ❖The loading procedure was performed by incubating different concentrations of BSA-V (1.0, 2.0,4.0,8.0)mg/ml with chitosan microparticles under mild agitation at room temperature for 15 min.
- ❖Loading efficiency (LE) of BSA-V on chitosan microparticles were detected in an indirect way by determining the free BSA-V remained in the supernatant after the performance of centrifuge, and the method was shown as following.
- ❖One millilitre of BSA loaded chitosan microparticles suspension was centrifuged at 14,000 rpm for 20 min and the amount of BSA in the supernatant was measured by BCA™ kit using a standard curve formulated through spectrophotometer.
- ❖ The supernatant of blank chitosan microparticles was adopted as the blank to correct the absorbance reading value of the BSA-loaded chitosan microparticles. The corrected optical density (OD) value was then used to calculate the concentration of BSA in the supernatant.

Preparation of alginate coated chitosan microparticles

- ❖ Alginate microparticles were prepared by dissolving sodium alginate in millli-Q water with mild agitation at room temperature.
- ❖ Then, alginate microparticles were dispersed into calcium chloride ($CaCl_2$) aqueous solution (pH = 7.0) at concentration of 0.5mM/litre.
- ❖ BSA-V loaded chitosan microparticles suspensions with different pH value were added drop wisely into sodium alginate solution containing calcium chloride of different pH value at different concentration (5.0 and 10.0) mg/ml under mild agitation for 10 min.
- ❖ Then the suspension was centrifuged at 3,500 rpm for 5 min, and the supernatant was discarded. (Two stage procedure).

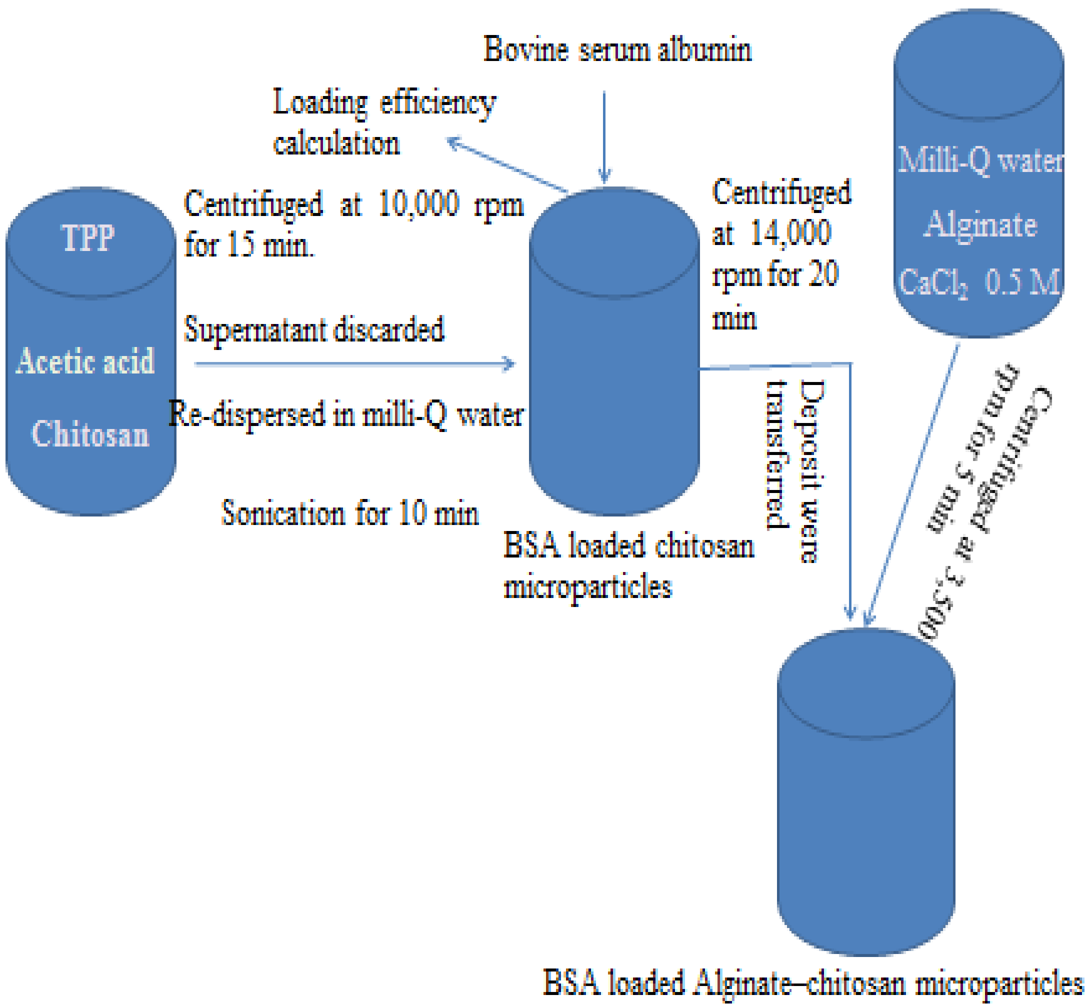


Fig.5 Schematic representation of method used in preparation of alginate coated chitosan microparticles.

$$LE(\%) = \frac{\text{total amount of BSA} - \text{free BSA}}{\text{total amount of BSA}} \times 100$$

Loading efficiency (LE %) was calculated by using above formula in which supernatant was considered for estimation of protein specrophotometrically.

Morphological characterization

Colloidal microparticles were first lyophilized for 24 hours. Then morphological characteristics of microparticles were examined by scanning electron microscope (SEM). For SEM, a specimen is normally required to be completely dry, since the specimen chamber is at high vacuum. Microparticles were sputtered with gold and maintained at room temperature for complete dryness before the observation.

In vitro **release and checking of protein degradation by SDS-PAGE**

After loading the protein (BSA) to the microparticles in vitro release was studied by using PBS (phosphate saline buffer) pH 7.4. One milliliter of microparticles suspension was first centrifuged and the deposit was incubated in 1 ml of phosphate buffer saline (PBS, pH7.4) in eppendorf tube (EP tube). Then, the EP tube was placed in an air shaker bath at 100 rpm/min (at 37°C) for *in vitro* release. At scheduled time, samples were centrifuged at 14,000 rpm for 20 min and the supernatant was replaced with fresh PBS (previously warmed to 37°C). The amount of BSA presented in the supernatant was determined by BCA™ kit using spectrophotometer. Then protein degradation was checked by SDS-PAGE(sodium dodecyl sulphate polyacrylamide gel electrophoresis) method in which release of protein was done in pH 2.0 by using HCl (hydrochloric acid) for 2 hours (37^{o}C) then sample was centrifuged at 14,000 rpm for 20 minutes and the supernatant was replaced with fresh PBS (previously warmed to 37°C). Schematic diagram of the release study is given in fig.6 below.

Particles containing BSA (1ml) centrifuged
at 14,000 rpm for 20 minutes in eppendorf
tube and deposit was obtained

PBS (pH 7.4)
(1 ml)

Deposit with 1ml PBS was incubated
in incubator shaker at 37^{o}C with 100 rpm

(After specific interval)

Samples were removed
and again centrifuged at 14000 rpm
For 20 minutes

Deposit
+
Fresh PBS 1 ml

Kept in incubator
Shaker for further studies
(37^{o}c, 100rpm)

Supernatant

Estimation for released
protein spectrophotometrically

Fig.6 Schematic representation of release study of BSA from Alginate coated chitosan microparticles.

In this research paper we have prepared alginate coated chitosan microparticles by two stage procedure in which first chitosan microparticles were prepared using ionic gelation technique (Hejazi and Amiji, 2003) by addition of sodium tripolyphosphate as a precipitating agent. Then BSA with isoelectric point (PI) of 4.8 was negatively charged when pH >4.8, which could easily absorb cationic chitosan microparticles at aqueous solution (pH 7.0) via electrostatic interactions. Thus BSA was used as a model protein for evaluation of the properties of the alginate coated chitosan microparticles. Simultaneously alginate microparticles were prepared by dissolving in milli-Q water and then 0.5 mM Calcium chloride solution was added to it in which calcium ion acts as a cross linking agent to strengthen and stabilize the particle . This completes the first stage of the procedure. In the second stage Alginate microparticles were added drop wisely on to the chitosan microparticles loaded with BSA protein.

Blank chitosan microparticles analysis

Sample 1250,130,140,150,1100,2250,230,240,250,2100 (see appendices) are the blank chitosan microparticles without loading of BSA were analysed in the zeta sizer to evaluate particle size, PDI and zeta potential by taking refractive index of chitosan 1.523.Some of the zeta sizer analysis graphs were given in fig 8-9.It was found that with increase in chitosan concentration the particle size increases. The particle size increased from 126.4nm to1321nm when chitosan concentration was increased from 2.5mg/ml to 10 mg/ml in case where chitosan was dissolved in 1% acetic acid (table 4). Similarly the particle size increased from 126.0 nm to 1266 nm when chitosan concentration was increased from 2.5mg/ml to 10 mg/ml in the case where chitosan was dissolved in 2% acetic acid as given in fig. 7 and table 5.Thus it can be also concluded that more the concentration of acetic acid less will be the particle size (but to a smaller extent), probably because chitosan were dissolved finely in more concentrated acetic acid. Respective PDI values of the formulations were evaluated.Polydispersity index (PDI), a term in polymer chemistry referring to the molecular weight distribution of polymers.

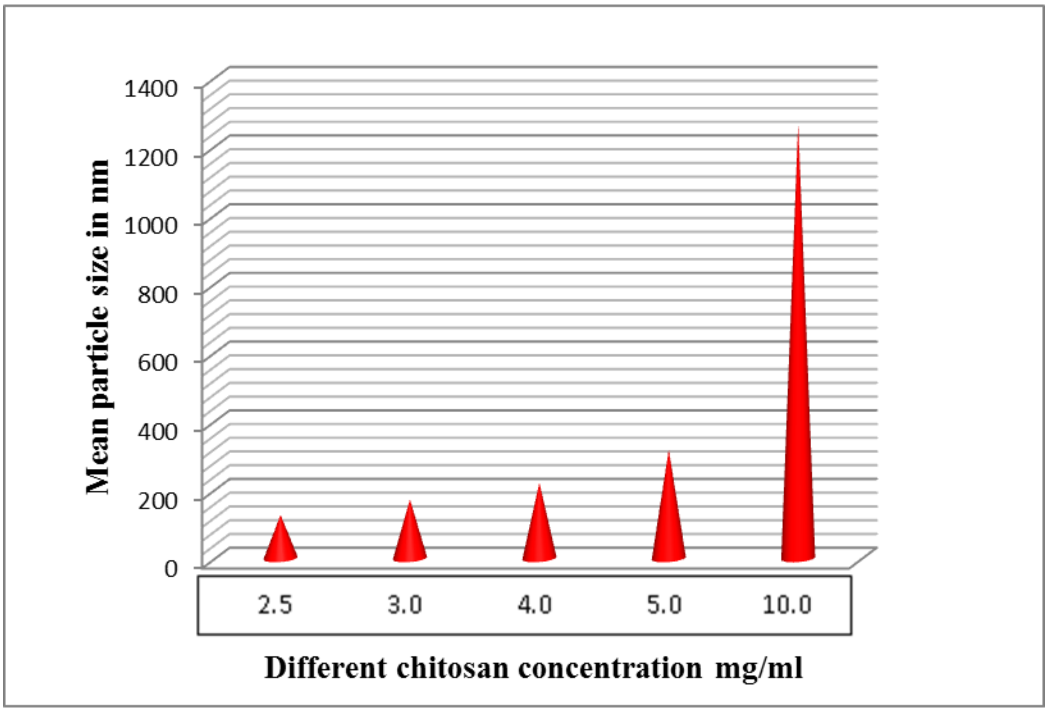


Fig 7. Effect of different concentratios of chitosan (dissolved in 2% acetic acid) on mean particle size.

Zeta potential is a scientific term for electro-kinetic potential (McNaught and Wilkinson, 1997) develops at the surface of the colloidal particles.in fig 9 shows the zeta potential readings and other distributions like voltage and current etc. The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate as outlined in the table 3.

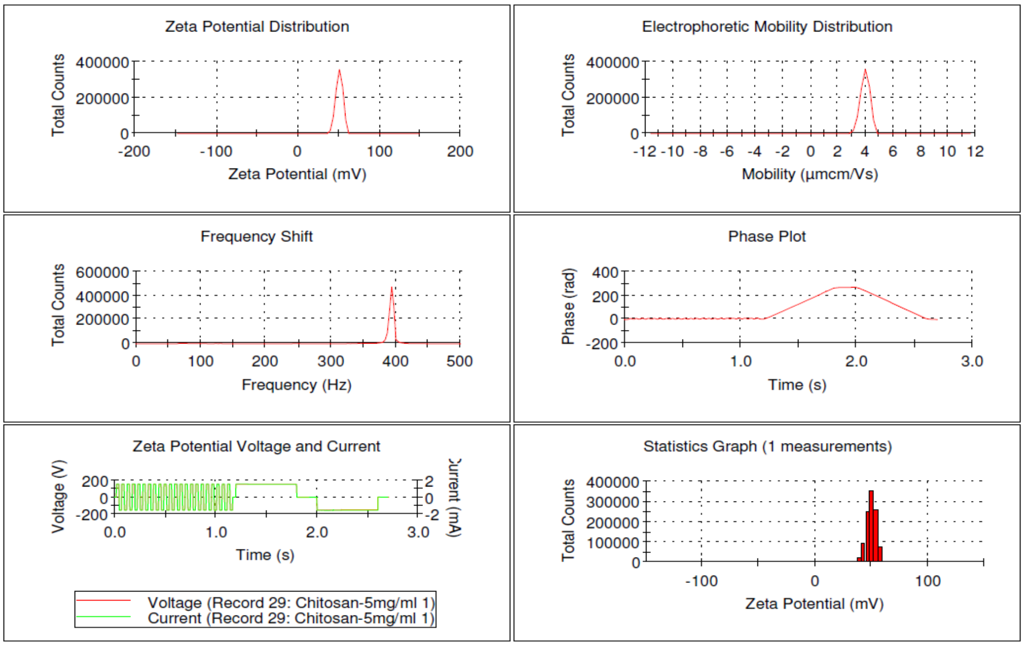
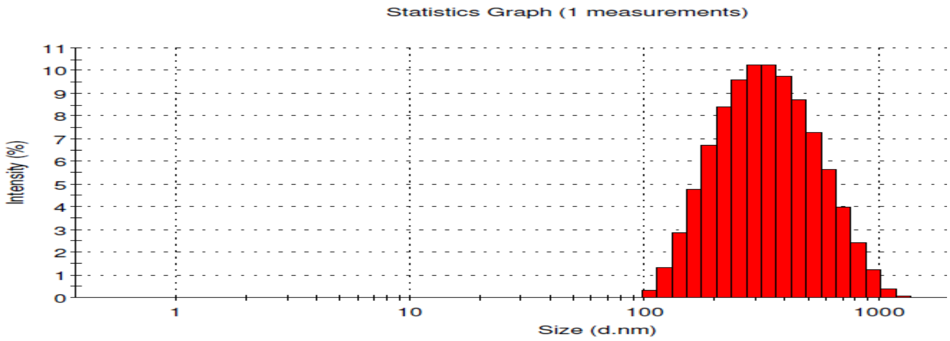


Fig.8 Zeta sizer analysis data with zeta potential of 5mg/ml



chitosan.

Fig. 9 Statistical analysis of particle size distribution of chitosan 5mg/ml.

Zeta potential [mV]	**Stability behaviour of the colloid**
From 0 to ±5,	Rapid coagulation or flocculation
From ±10 to ±30	Incipient instability
From ±30 to ±40	Moderate stability
From ±40 to ±60	Good stability
More than ±61	Excellent stability

Table 3.Zeta potential and colloid stability behaviours derived from Zeta Potential of Colloids in Water and Waste Water", ASTM Standard D 4187-82, American Society for Testing and Materials, 1985.

Zeta potential of maximum samples were found to be from +30 to +60(samples loaded with 1mg/ml and 2mg/ml BSA) i.e. chitosan colloid particles loaded with 1mg/ml and 2mg/ml BSA were having moderate to good stability. On the other hand chitosan colloid particles loaded with 4mg/ml and 8mg/ml BSA, zeta potential were varies from +10 to +30 (maximum case) i.e. chitosan colloid particles loaded with 4mg/ml and 8mg/ml BSA were having incipient stability. In some case of chitosan microparticles loaded with 8mg/ml BSA, zeta potential was less than +10 means sample were having rapid coagulation or flocculation properties.

Procedure of BSA loading and Effect of BSA concentration on chitosan microparticles

The chitosan microparticles were incubated in aqueous solution with different BSA concentrations at a relatively mild condition to obtain suitable loading efficiency LE).Cationic microparticles can easily absorb anionic protein or DNA via electrostatic interaction (Illum *et al*., 2001; Cui and Mumper, 2001).Cationic chitosan microparticles prepared in this work had a potent capacity to absorb the model anionic protein (BSA) in aqueous solution (pH 7.0) via electrostatic interaction. The obtained chitosan microparticles at different concentrations were adopted to evaluate particle size, zeta potential, loading efficiency. The loading efficiency was calculated using BCA standard graph for BSA protein by using spectrophotometer (fig 10).

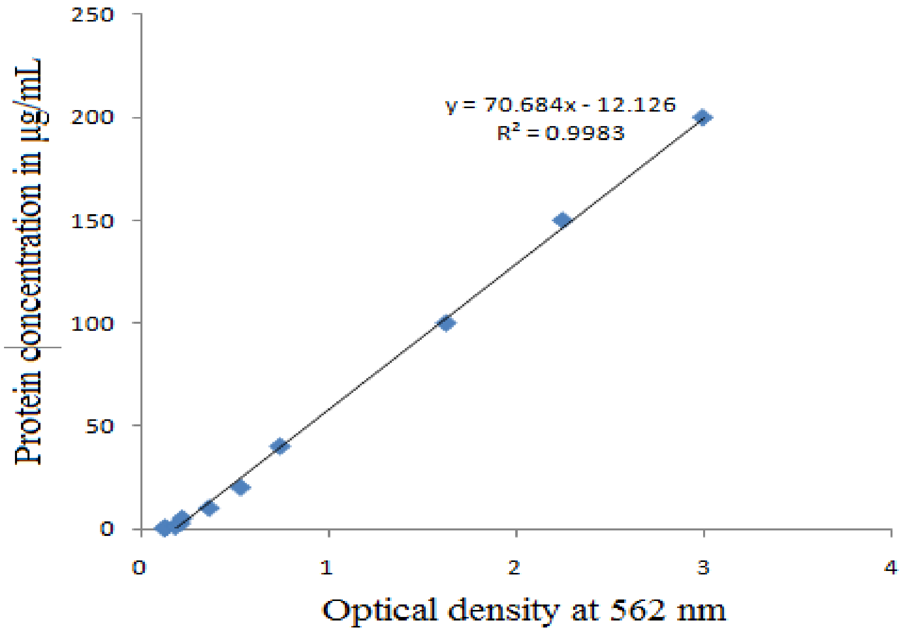


Fig 10 .BCA standard graph using BCA™ kit

As presented in Table 4 and 5 effect of BSA concentration on the properties of chitosan microparticles were investigated. The mean diameter of chitosan microparticles increased accompanied with decrease in zeta potential when BSA concentration increased from 1 mg/ml to 8 mg/ml. This might be attributed to the fact that negatively charged BSA absorbed onto chitosan microparticles and neutralized part of zeta potential of chitosan microparticles resulted in increase of particle size and decrease of zeta potential (Xu and Du, 2003).It was seen that when BSA concentration was less than 2mg/ml or 2mg/ml then the loading efficiency was more as compared to other formulations with BSA concentration 4mg/ml or 8 mg/ml. This interesting phenomenon might be attributed to the saturated absorption was achieved as BSA concentration at about 2 mg/ml, the more addition of BSA was seldom adsorbed onto chitosan microparticles which leaded to the great decrease in loading efficiency (Xing *et al*., 2008).The loading efficiency of 141, 151, 1101, 1102, 251, 252, 2101 and 2102 were found to be more than 60% while sample 241 was found to be 59%(table 2 and 3).The mean particle size of the formulation 1101, 1102.2101 and 2102 were more than 1350 nm. Thus, these formulations were not taken into account because of their large particle size. Here in this article formulation with chitosan 4mg/ml, 5mg/ml were taken as standardised particles to coat with alginate microparticles. A comparative graphical presentation of the variation in loading efficiency due to change in concentrations of different parameters like BSA concentration, Chitosan concentration and also to small extent the acetic acid concentration is given in fig 11.

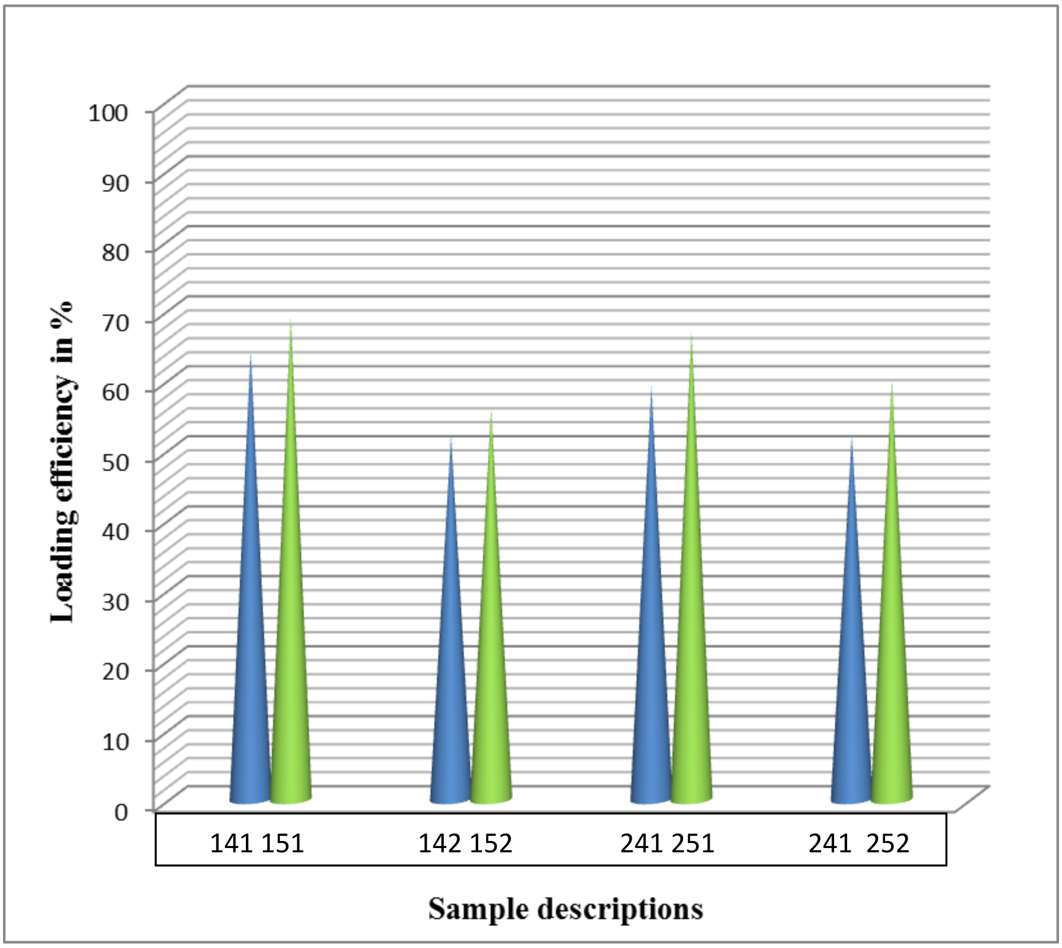


Fig 11. Effect of different BSA and chitosan concentration on loading efficiency (LE).

Table 4.Formulations of chitosan microparticles with 1% acetic acid and their mean particle size, PDI and zeta potential.

Sl.No	Sample name	pH	Mean particle size	PDI	Zeta potential	Loading efficiency (%)
1.	1250	3.48	126.4	0.507	+43.9	---
2.	1251	--	215.0	0.337	+33.3	44.0
3.	1252	--	243.8	0.410	+24.5	32.0
4.	1254	--	256.1	0.370	+14.2	24.0
5.	1258	--	289.6	0.432	+9.4	17.0
6.	130	3.62	174.1	0.383	+46.2	---
7.	131		259.8	0.447	+35.5	47.0
8.	132	--	294.5	0.387	+26.6	35.0
9.	134	--	327.0	0.471	+16.5	26.0
10.	138	--	353.9	0.337	+10.9	18.0
11.	140	3.82	236.5	0.468	+53.5	---
12.	141	--	309.4	0.337	+36.1	64.0
13.	142	--	326.7	0.391	+28.5	52.0
14.	144	--	347.3	0.379	+17.3	40.0
15.	148	--	379.9	0.399	+11.1	29.0
16.	150	3.97	316.4	0.476	+45.4	---
17.	151	--	402.3	0.447	+36.8	69.0
18.	152	--	419.0	0.509	+29.4	56.0
19.	154	--	443.4	0.534	+20.7	42.0
20.	158	--	478.7	0.584	+11.9	31.0
21.	1100	4.19	1321.0	0.802	+54.9	---
22.	1101	--	1357.0	0.900	+43.5	66.0
23.	1102	--	1378.1	0.943	+34.9	61.0
24.	1104	--	1398.5	0.954	+31.2	50.0
25.	1108	--	1402.3	0.977	+23.9	44.0

--Not checked

---No loading of protein

Table 5.Formulations of chitosan microparticles with 2% acetic acid and their Mean particle size, PDI and zeta potential.

Sl.No	Sample name	pH	Mean particle size	PDI	Zeta potential	Loading efficiency (%)
26.	2250	3.34	126.0	0.455	+40.2	---
27.	2251	--	213.6	0.339	+34.3	48.0
28.	2252	--	243.8	0.440	+23.6	30.0
29.	2254	--	256.9	0.370	+14.4	25.0
30.	2258	--	283.4	0.439	+9.9	19.0
31.	230	3.40	170.9	0.445	+46.2	---
32.	231	--	269.3	0.497	+35.8	50.0
33.	232	--	299.8	0.387	+26.0	38.0
34.	234	--	329.1	0.481	+16.5	30.0
35.	238	--	358.9	0.387	+10.9	22.0
36.	240	3.49	219.9	0.456	+48.0	---
37.	241	--	309.9	0.357	+34.9	59.0
38.	242	--	336.7	0.399	+28.8	52.0
39.	244	--	348.8	0.379	+19.8	41.0
40.	248	--	372.2	0.390	+11.9	29.0
41.	250	3.55	315.8	0.384	+45.4	---
42.	251	--	400.3	0.497	+34.8	67.0
43.	252	--	419.8	0.589	+29.4	60.0
44.	254	--	448.9	0.584	+20.2	42.0
45.	258	--	473.9	0.584	+13.9	31.0
46.	2100	3.81	1266.0	0.905	+54.2	---
47.	2101	--	1352.0	0.899	+48.5	66.0
48.	2102	--	1373.1	0.948	+34.9	61.0
49.	2104	--	1390.8	0.959	+34.2	49.0
50.	2108	--	1400.8	0.979	+23.9	41.0

--Not checked

---No loading of protein

Blank Alginate microparticles analysis

Two samples of alginate microparticles having 5mg/ml and 10 mg/ml concentrations were prepared by dissolving Alginate in milli-Q water with mild agitation by magnetic stirrer in room temperature and then alginate microparticles were dispersed into calcium chloride ($CaCl_2$) aqueous solution (pH = 7.0) at concentration of 0.5mM/litre. Particles were analysed in zeta sizer to evaluate particle size, PDI and zeta potential as given in table 6 and fig 12-13.As alginate is anionic thus zeta potential was found negative. For both the concentrations zeta potentials found to be more than -63 i.e. Alginate microparticles were showing excellent stability. The particle size of 5mg/ml and 10 mg/ml alginate solution were 1147nm and 3914 nm respectively. Due to larger particle size 10 mg/ml alginate solution were not taken into consideration for further studies to formulate alginate coated chitosan microparticles formation.

Table 6.Formulations of alginate microparticles with mean particle size, PDI and zeta potential.

Sl No.	Concentration of Alginate mg/ml	Mean particle size in nm	PDI	Zeta potential(mV)
1.	5.0	1157.0	0.446	-63.1
2.	10.0	3914.0	0.683	-63.3

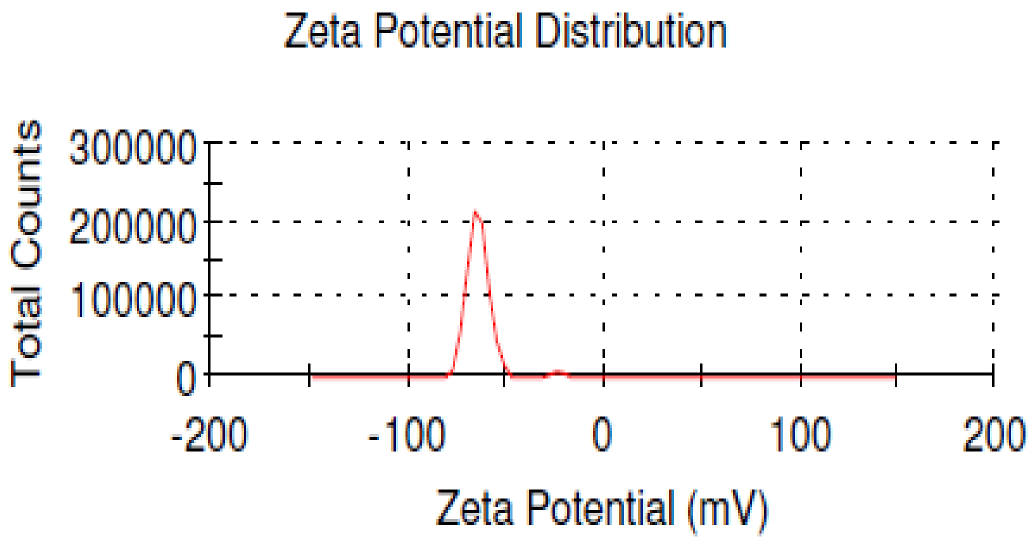


Fig. 12.Zeta potential distribution of alginate microparticles.

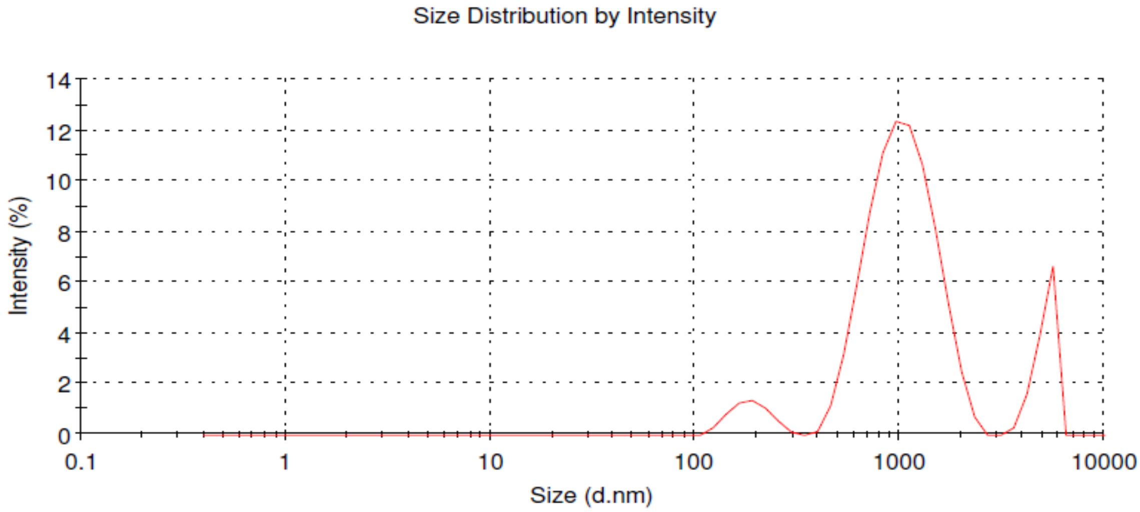


Fig 13 Particle size distribution of alginate 5mg/ml microparticles.

Alginate coated BSA loaded chitosan microparticles analysis

Due to the electrostatic interaction between the positively charged $-NH_3^+$ of chitosan microparticles and negatively charged $-COO^-$ of alginate, cationic BSA-loaded chitosan microparticles could be easily modified with anionic alginate in aqueous solution via electrostatic interaction (Fan *et al*., 2006). However, the addition of anionic alginate to cationic BSA loaded chitosan microparticles would replace some of BSA absorption on the chitosan microparticles due to the competitive electronic interaction (Borges *et al*., 2005). Alginate were coated to the BSA loaded chitosan microparticles having higher stability and greater LE like samples 141,142,151,152,241,242,251 and 252.After coating of alginate 5mg/ml to all the samples the zeta potential evaluated between -20 to -30 which shown incipient stability properties according to table 7.The mean particle size of the formulations 1415, 1425, 2415and 2425 were found to be in between 1400 nm to 1425 nm while formulations 1515, 1525, 2515and 2525 were having particle size in between 1547nm to 1570 nm.A comparative account of particle size of different alginate coated chitosan microparticles are given in fig 14- 16 .The zeta potential value of the different alginate coated chitosan microparticles were in between -20 to -30 showing incipient stability.

Table 7.Formulations of alginate coated chitosan microparticles with mean particle size, PDI and zeta potential.

Sample code	Mean particle size	PDI	Zeta potential	After coating Alginate 5 mg/ml Sample	Mean particle size	PDI	Zeta potential
141	309.4	0.337	+36.1	1415	1402.5	0.409	-26.7
142	326.7	0.391	+28.5	1425	1415.7	0.468	-22.5
151	402.3	0.447	+36.8	1515	1554.0	0.497	-26.1
152	419.0	0.509	+29.4	1525	1567.9	0.543	-21.4
241	309.9	0.357	+34.9	2415	1405.9	0.413	-27.8
242	336.7	0.399	+28.8	2425	1422.7	0.439	-23.9
251	400.3	0.497	+34.8	2515	1547.2	0.556	-27.3
252	419.8	0.589	+29.4	2525	1569.0	0.599	-22.7

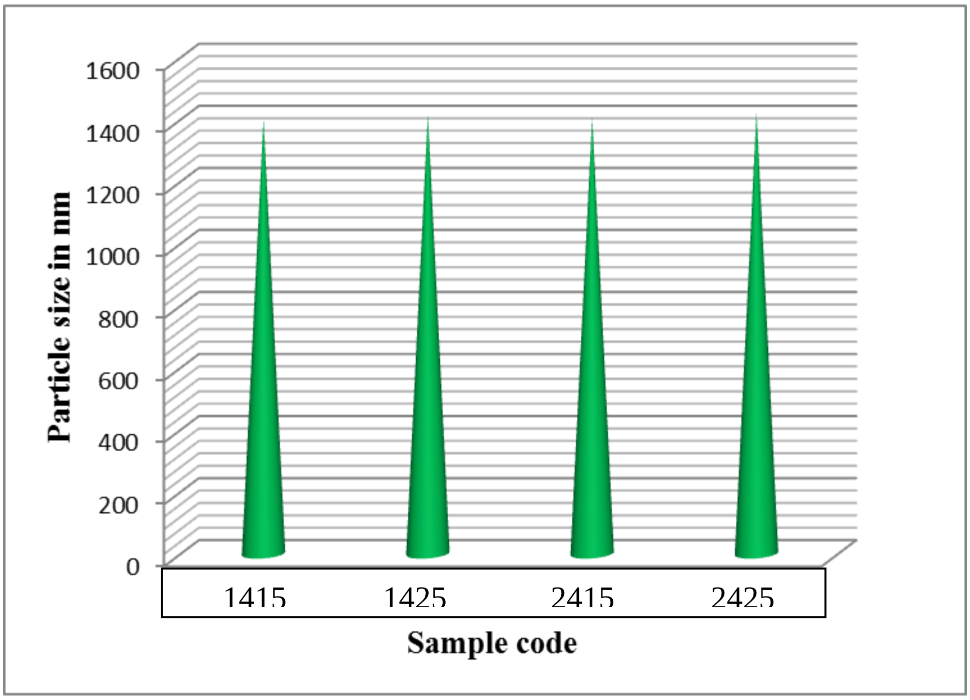


Fig14.Comparative analysis of alginate coated chitosan microparticles where chitosan concentration was 4mg/ml and different BSA was used.

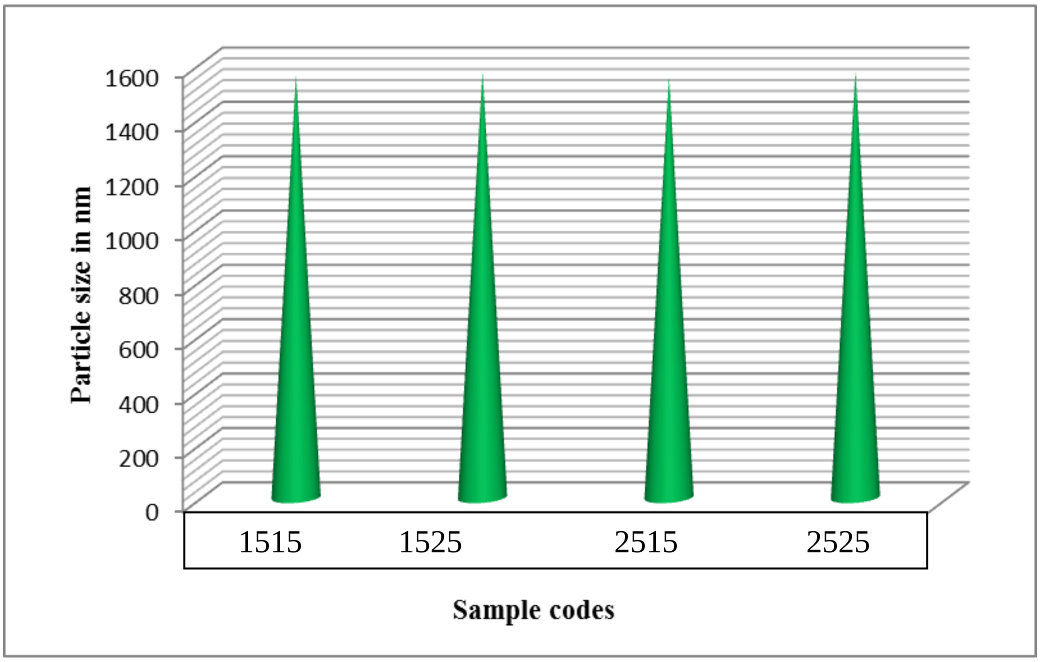


Fig15.Comparative analysis of alginate coated chitosan microparticles where chitosan concentration was 5mg/ml and different BSA was used.

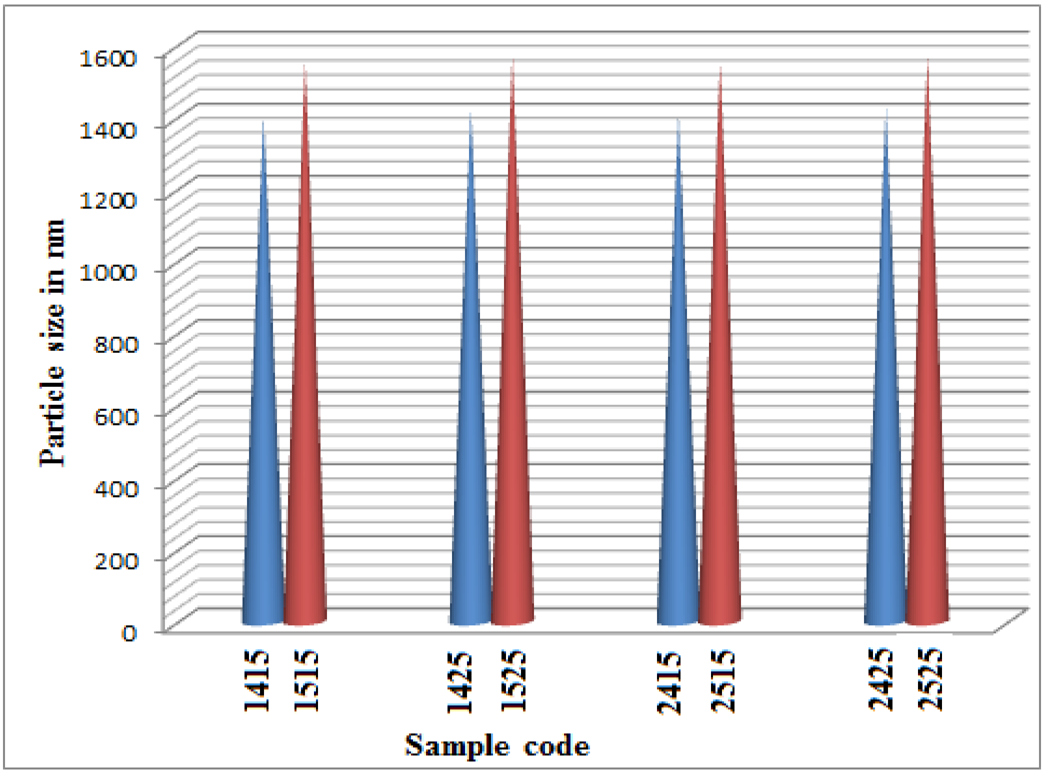


Fig16.Comparative analysis of alginate coated chitosan microparticles where chitosan concentration was 4mg/ml and 5mg/ml with different BSA concentration was used.

***In vitro* Protein release study**

The release profiles of BSA from uncoated chitosan and alginate coated chitosan microparticles in phosphate buffer (PBS, pH7.4). As depicted in Fig 17-20, the initial burst release of BSA from uncoated chitosan microparticles 141,142,151 and 152 was about 87.5%,87.9%,86.2% and 85.3% respectively occurred in the first 0.5 hour, followed by release of 97.1% ,97%,96.9% and 96.5% respectively in 48 hours. The burst release might be attributed to the fact that BSA macromolecules were loosely bound onto chitosan microparticles by ionic interaction which could be easily desorbed at ionic environment (Chen *et al*., 2007). However, alginate modified chitosan microparticles could increase the stability of chitosan microparticles in the PBS at 37°C which resulted in extended release of BSA, in these formulations like 1415, 1425, 1515 and 1525 only about 21.1%, 21.2%, 20.9% and 21.2% of BSA released in 0.5 hour respectively and 48 h later, there was still about 50% of BSA retained in the alginate coated chitosan microparticles.

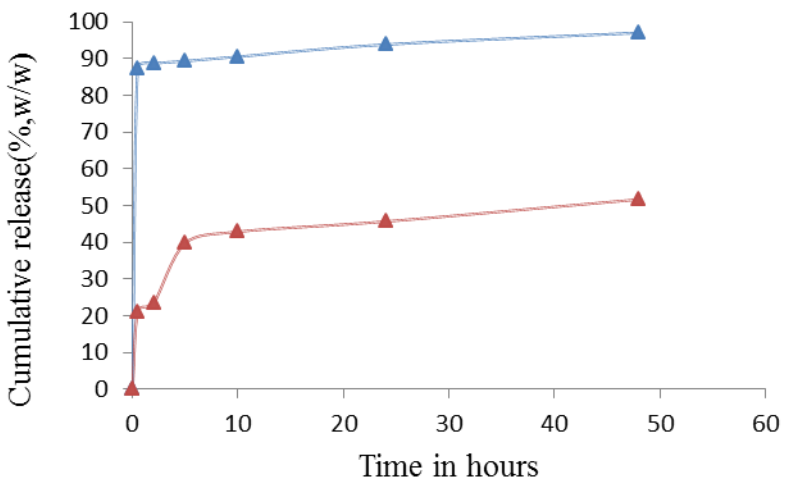


Fig 17. Comparative release study of sample 141 (up) and sample 1415 (down).

The total ratio of BSA released from the alginate coated chitosan microparticles in 48 h was much less than that observed from uncoated chitosan microparticles. The longer release time and slower release rate of BSA from alginate coated chitosan microparticles might be explained by that there are strong interaction between polymers (chitosan and alginate) and BSA (Coppi *et al*.,2001).

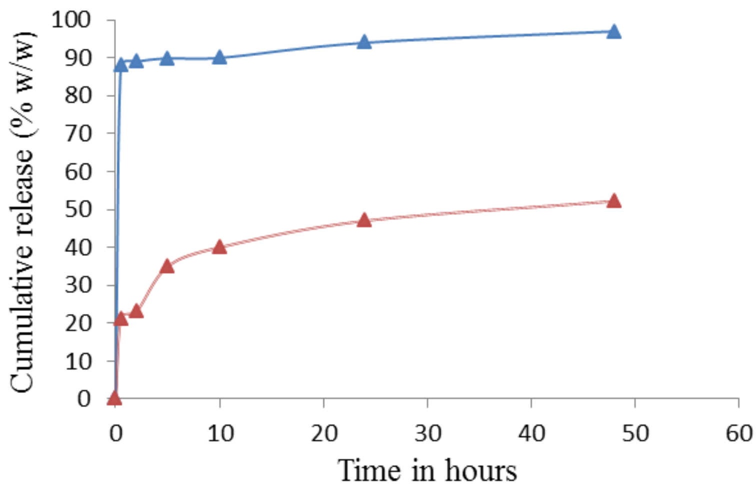


Fig18.Comparative release study of sample 142 (up) and sample 1425 (down).

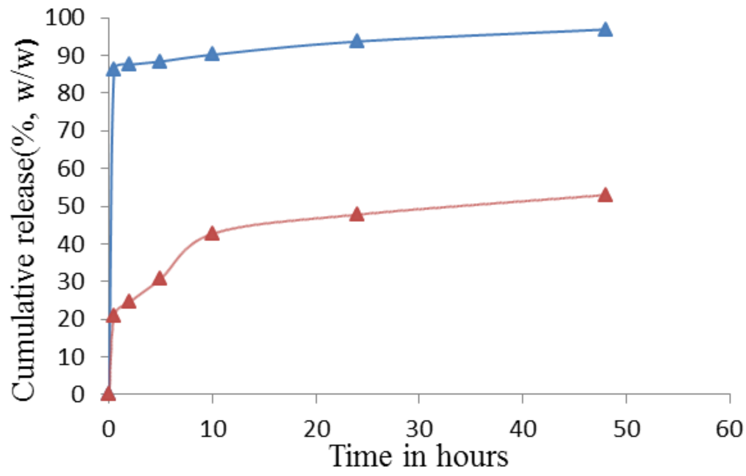


Fig 19. Comparative release study of sample 151(up) and sample 1515(down).

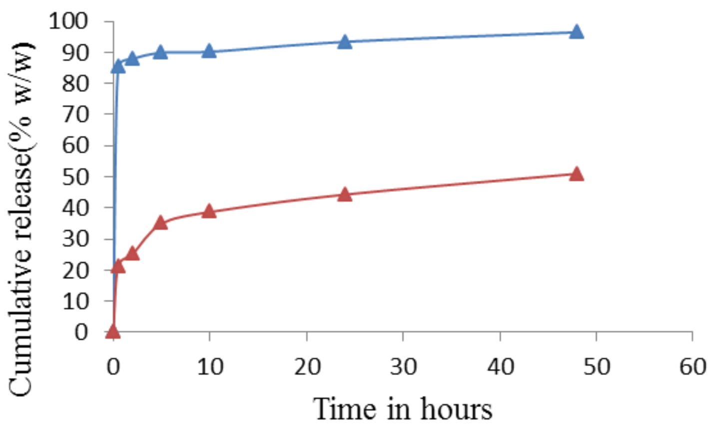
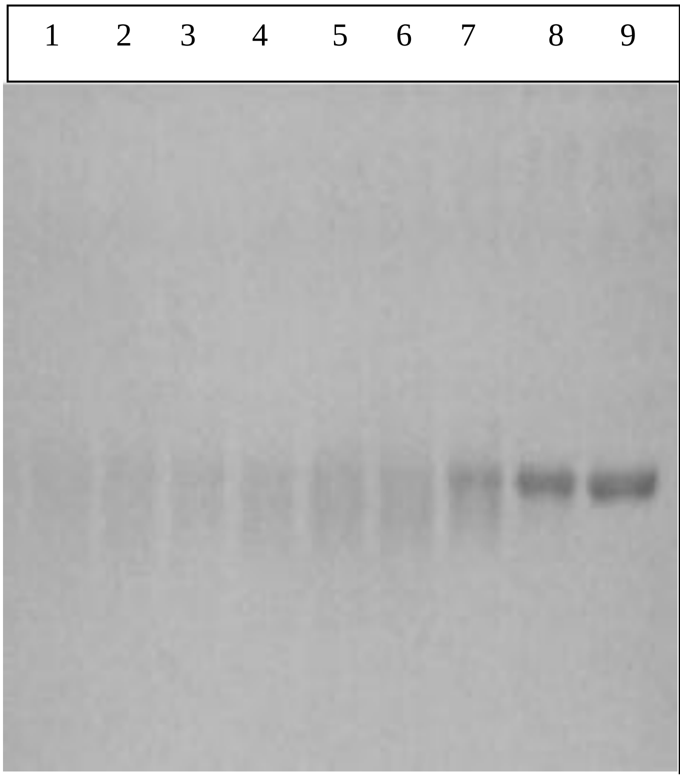


Fig 20.Comparative release study of sample 152(up) and sample 1525(down).

The total ratio of BSA released from the alginate coated chitosan microparticles in 48 h was much less than that observed from uncoated chitosan microparticles. The longer release time and slower release rate of BSA from alginate coated chitosan microparticles might be explained by that there are strong interaction between polymers (chitosan and alginate) and BSA (Coppi *et al*., 2001). Additionally, the presence of alginate layer could slow down the diffusion of BSA from the system which also prolong the release time of BSA from system. But, due to both the degradation and erosion of matrix, the BSA might be released from the alginate/BSA/chitosan formulation in an extended profile (Anal et al., 2003). This release behaviour studies indicated that coating of alginate onto chitosan microparticles could improve the stability of chitosan microparticles in PBS and modify the release behaviour of BSA from these alginate coated chitosan microparticles.

Detection of Acidic degradation protection by SDS-PAGE

In this paper, I have tried to design an oral drug delivery system for protein. As we know, pH value of gastric fluid is approximately 2 which will destroy the integrity and structure of antigens (protein, DNA, etc.) after the oral administration without any protection (Xing et al., 2008). Thus, it is very important to develop a protein carrier based on chitosan and alginate that can effectively avoid the acidic degradation of encapsulated protein before it reached the target site. Here, BSA released from uncoated and alginate coated chitosan microparticles under acidic medium (pH2.0) and PBS medium (pH7.4) was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).



Lane 1: 151 in 0.01M HCl

Lane 2: 252 in 0.01M HCl

Lane 3: 152 in 0.01M HCl

Lane 4:251 in PBS for 24 hr

Lane 5:151 in PBS for 24 hr

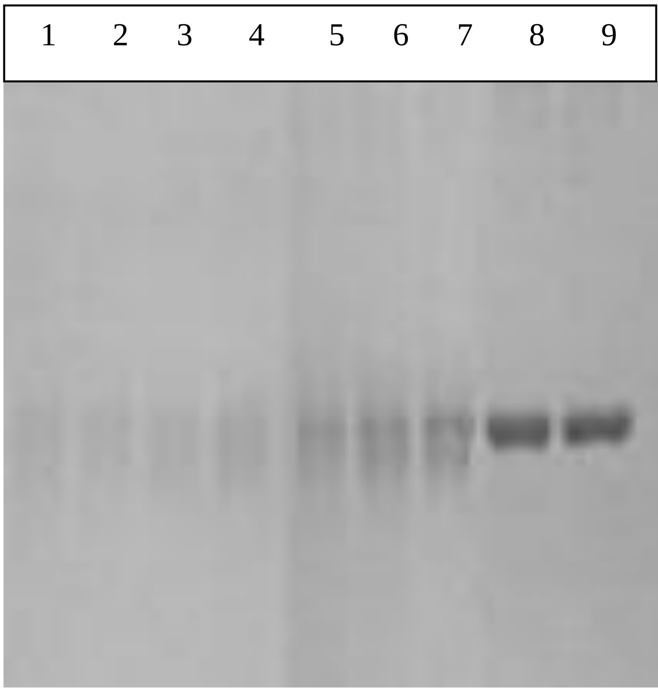
Lane 6:252 in PBS for 24 hr

Lane 7:152 in PBS for 24 hr

Lane 8: Control BSA 2mg/ml

Lane 9: Control BSA 4mg/ml

Fig. 21.SDS-PAGE for BSA loaded alginate chitosan microparticles for analysis of protein degradation.



Lane 1: 1515 in 0.01M HCl

Lane 2: 2525 in 0.01M HCl

Lane 3: 1525 in 0.01M HCl

Lane 4:2515 in 0.01M HCl

Lane 5:1515 in PBS for 24 hr

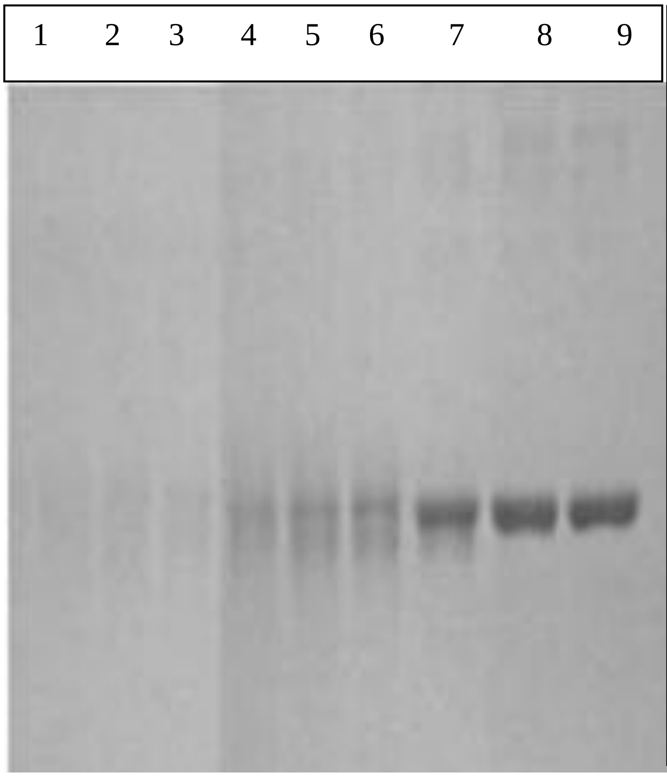
Lane 6:2525in PBS for 24 hr

Lane 7:1525 in PBS for 24 hr

Lane 8: Control BSA 2mg/ml

Lane 9: Control BSA 4mg/ml

Fig. 22. SDS-PAGE for BSA loaded uncoated chitosan microparticles for analysis of protein degradation.



Lane 1: 151 in 0.01M HCl

Lane 2: 252 in 0.01M HCl

Lane 3: 152 in 0.01M HCl

Lane 4:1515 in 0.01M HCl

Lane 5: 1525 in 0.01M HCl

Lane 6: 2525 in 0.01M HCl

Lane 7: 1525 in PBS for 24 hr

Lane 8: Control BSA 2mg/ml

Lane 9: Control BSA 4mg/ml

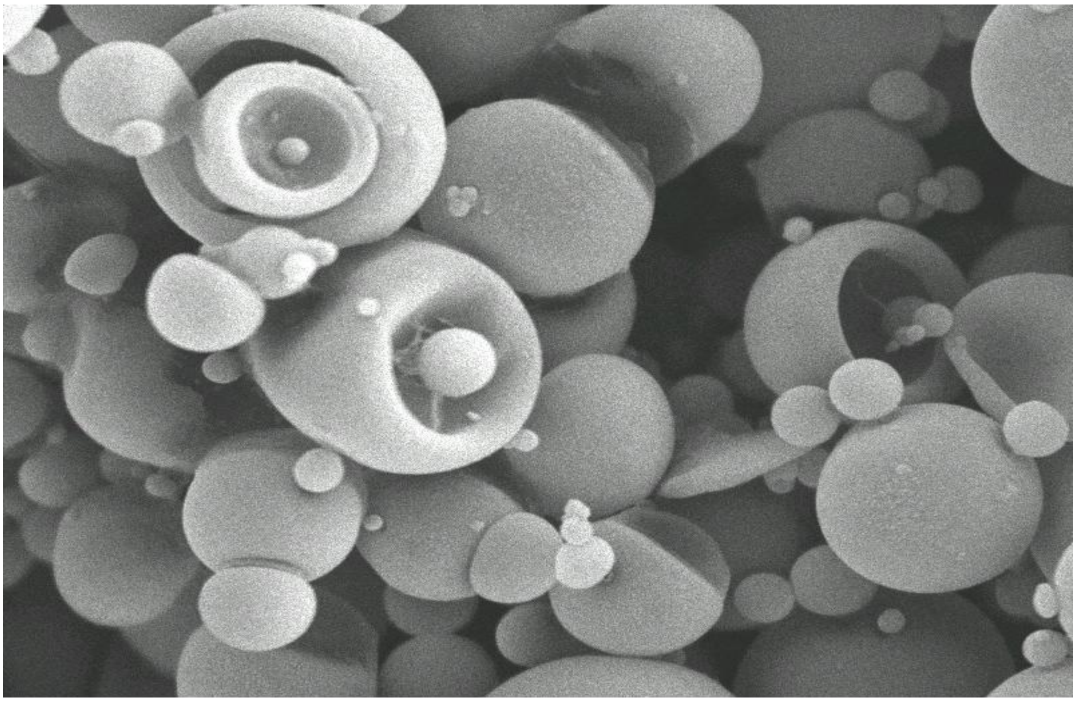
Fig. 23. SDS-PAGE for BSA loaded uncoated chitosan and alginate coated chitosan microparticles for analysis of protein degradation.

According to Fig.23, BSA 2mg/ml and 4mg/ml (Lane 8 and Lane 9 respectively) were taken as control and the different formulations of BSA loaded alginate chitosan microparticles were incubated with PBS (pH7.4) for 24 h exhibited a clear band (Lane 4, Lane 5, Lane 6 and Lane 7) at about at same distance as control (Lane 8 and Lane 9). However, BSA pre-treated with 0.01 M HCl for 2 hours then incubated with PBS for 24 hours had a faint band (Lane 1 ,Lane 2 and Lane 3)at same distance as control (Lane 8 and Lane 9) which indicated the serious degradation or hydrolysis of BSA in acidic medium. The SDS-PAGE gel banding patterns of BSA released from uncoated and alginate coated chitosan microparticles with HCl (pH2.0) pre-treatment for 2 h and then sustained release in PBS for 24 h are shown in Lane 1 and Lane 2, respectively. It is seen that BSA from uncoated chitosan microparticles had a very weak band at control (Lane 1) which indicated that the BSA underwent hydrolysis/degradation in spite of presence of chitosan microparticles (pH2.0).

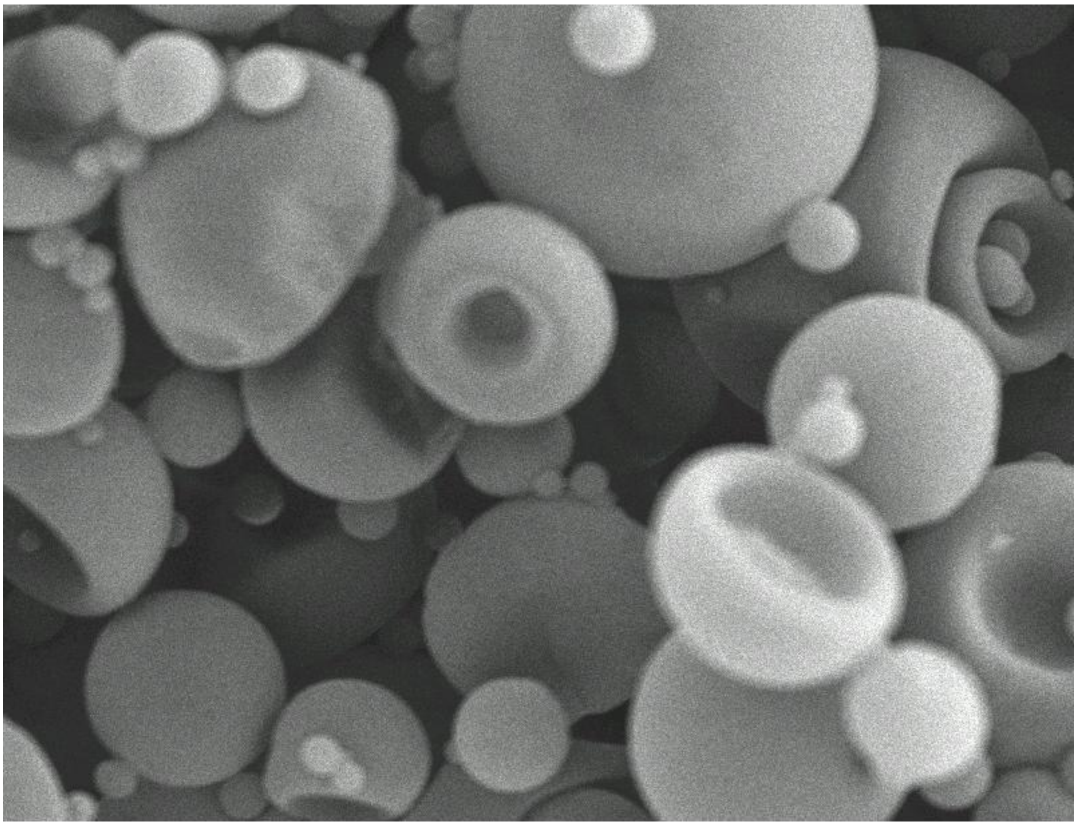
Meanwhile, we also observed the flocculent precipitate of system with the addition of sodium hydrate to BSA loaded chitosan microparticles which implied the dissolution of chitosan microparticles in acidic condition (pH2). However, BSA from alginate coated chitosan microparticles had a clear band at about control (Lane 2), which implied that alginate coating of chitosan microparticles could effectively protect BSA from hydrolysis/degradation at acidic medium for at least 2 h. So, the obtained alginate coated chitosan microparticles might be an effective oral protein delivery.

Characterization of chitosan microparticles

The obtained chitosan microparticles and alginate coated BSA loaded chitosan microparticles were characterized by SEM and laser diffraction. Two typical scanning electron microphotographs BSA loaded chitosan microparticles were presented in fig 24 (a) and (b).and shown that some of the particles were more than 1μm in diameter. Just similar peaks of particle size were also observed in Malvern zeta sizer instrument. Some of the particles were observed as 0.1 μm in size but most of the particles were in between 200-600 nm in size were observed. It was also observed that some small chitosan microparticles fused into large ones. The result detected by Malvern Instrument indicated that the average size of chitosan microparticles was about 315 nm (5mg/ml chitosan).



(a)



(b)

Fig.24 (a) and (b) SEM images of BSA loaded chitosan microparticles.

The prepared alginate coated chitosan microparticles, with mean diameter of about 1.5 µm, were suitable for oral administration. Alginate coating onto surface of chitosan microparticles could modulate the release behaviour of BSA from alginate coated chitosan microparticles and it could effectively protect model protein (BSA) from degradation against acidic medium (pH 2) *in vitro* at least for 2 hours based on the information demonstrated, by SDS-PAGE analysis of the protein degradation. Thus, it can be concluded that prepared alginate coated chitosan microparticles are cost effective oral drug delivery system that can be used as a carrier for proteinaceous drugs. It can effectively release the encapsulated proteinaceous drugs and protect its degradation from GIT barriers for a longer period of time.

1. Agarwal, V. and Khan, M.A., 2001. Current status of the oral delivery of insulin. Pharm. Technol. 10, 76–90.

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